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ORIGINAL RESEARCH

Obinutuzumab activates FcγRI more potently than other anti-CD20 antibodies in chronic lymphocytic leukemia (CLL)

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ABSTRACT

Treatment with monoclonal antibodies has revolutionized clinical medicine, especially in the fields of cancer and immunology. One of the oldest antibodies, which is widely used for the treatment of lymphomas and autoimmune diseases, is the anti-CD20 antibody rituximab. In recent years, new antibodies against CD20 have been developed including ofatumumab and obinutuzumab. An important mechanism of action of therapeutic monoclonal antibodies is activation of immune cells via Fc receptors (FcγRs). However, surprisingly, little is known about triggering of FcγRs by different therapeutic antibodies in general and anti-CD20 antibodies in particular. Here we establish a reporter assay to assess whether a particular antibody activates a certain Fc receptor. Using this assay we corroborated previous reports demonstrating obinutuzumab's ability to highly activate FcγRIIIa (CD16a). Importantly, we discovered that obinutuzumab also activates FcγRI (CD64) significantly more than rituximab and ofatumumab in response to chronic lymphocytic leukemia (CLL) cells obtained from patients. Mechanistically we show that this is due to the lack of FcγRIIb-mediated internalization of obinutuzumab following binding to CD20. Moreover, we show that obinutuzumab induces increased phagocytosis by primary macrophages in an FcγRI-dependent manner. Beyond the discovery of a new mechanism of obinutuzumab activity, the reporter assay can be applied to other therapeutic antibodies and may assist in developing antibodies with improved immunological properties.

Introduction

The anti-CD20 monoclonal antibody rituximab has significantly improved the treatment of B-cell lymphoproliferative diseases[1] as well as that of autoimmune diseases.[2] Rituximab is currently part of the backbone treatment regimen of many B-cell lymphoproliferative diseases including diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL).[3] Rituximab also constitutes an important component in the treatment of several autoimmune diseases, especially autoimmune hemolytic anemia and immune thrombocytopenic purpura (ITP).[4,5]

In recent years, new antibodies against CD20 have been developed, notably ofatumumab and obinutuzumab.[6] Both antibodies have been used for the treatment of CLL with possible clinical advantage of obinutuzumab over rituximab.[7,8] Obinutuzumab was also recently approved for the treatment of relapsed/refractory follicular lymphoma.[9] Two other anti-CD20 antibodies, ibritumomab and tositumomab, were conjugated to a radioactive substance to enhance their cytotoxic activity, and additional anti-CD20 antibodies are currently being studied in clinical trials.[6] In this study, we focused on the three anti-CD20 antibodies which are in clinical use in CLL: rituximab, ofatumumab and obinutuzumab.

Understanding how the various anti-CD20 antibodies exert their clinical outcome is of major importance.[10] Two main structural properties can explain the clinically observed differences of the various anti-CD20 antibodies. The first is the specific recognition of the antigen by the antibody, i.e. different CD20 epitopes are recognized by the different antibodies. In addition, previous studies demonstrated that some anti-CD20 antibodies (e.g., rituximab and ofatumumab) are able to localize CD20 into lipid rafts, while others (e.g., obinutuzumab) are not, referred as "type I" and "type II" antibodies, respectively.[11] This separation is not merely limited to CD20 localization but it is also associated with several other features, such as reduced recruitment of the complement system[12] and less internalization by type II antibodies.[13,14]

The second property is related to differences in the Fc portion of the various antibodies, which could affect complement recruitment and activation of Fc receptors on immune cells. Recognition of the antibody by various Fc receptors may be determined by the specific Fc isotype, specific amino acid sequences or specific glycosylations of the Fc segment. For example, the Fc segment of obinutuzumab has been glycoengineered so that the oligosaccharides attached to asparagine 297 (Asn^{297}) in the Fc region are non-

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fucosylated in order to enhance the binding of the antibody to Fcγ receptor IIIa (FcγRIIIa, CD16a).[12]

Surprisingly, the interactions of various therapeutic antibodies with various Fc receptors have not been fully elucidated. Moreover, the available data on the interaction of different anti-CD20 antibodies and human Fc receptors is based on affinity tests, mainly surface plasmon resonance (SPR),[12] and not on assays which measure the activation of Fc receptors.

Here we implemented a reporter assay to study whether certain anti-CD20 antibodies activate a particular Fc receptor. Using this system, we were able to demonstrate that obinutuzumab leads to increased activation of FcγRI (CD64) as compared to rituximab or ofatumumab.

Results

A reporter assay to study interactions of anti-CD20 antibodies with human Fcγ receptors (FcγRs)

To study which of the FcγRs can be activated, individually, by various anti-CD20 antibodies we stably transfected mouse BW5147 cells (BW cells) with chimeric FcγRs. These chimeric FcγRs included the extracellular part of a given FcγR fused to the transmembrane and cytoplasmic segments of the mouse CD3ζ chain. Activation of a specific FcγR results in secretion of mouse interleukin-2 (mIL-2) which can be detected by ELISA (Fig. 1A).

We transduced BW cells with either of the following human FcγR-CD3ζ chimeras: FcγRIIIa (CD16a, low and high affinity variants 158F and 158V respectively), FcγRIIa (CD32 a, low

and high affinity variants, 131R and 131H respectively), and FcγRI (CD64). We verified the expression of the extracellular part of the FcγRs in the transfected BW cells by flow cytometry (Fig. 1B).

Obinutuzumab leads to increased activation of FcγRIIIa and FcγRI in response to Raji cells

We subsequently used this BW cell reporter system to study the differential activation of FcγRs by three anti-CD20 antibodies which are in clinical use: rituximab, ofatumumab and obinutuzumab. Obinutuzumab has been glycoengineered to have enhanced affinity to the two variants of FcγRIIIa.[12] To test whether our reporter system is able to recapitulate these previous observations, we first studied the BW cells expressing the two variants of FcγRIIIa (158F and 158V, low and high-affinity respectively). We pre-incubated Raji cells, which express CD20, with rituximab, ofatumumab or obinutuzumab. The mIL-2 level with each antibody was normalized to the mIL-2 level of the control cells (transfected BW cells incubated with Raji cells without any antibody, defined as one). The pre-incubation of each of the three anti-CD20 antibodies with Raji cells resulted in secretion of mIL-2 significantly above the background level (Fig. 2A, the background level is marked with a horizontal line). More importantly, obinutuzumab activated the two variants of FcγRIIIa more efficiently than rituximab and ofatumumab (Fig. 2A). As a negative control, we repeated this experiment with BW cells expressing an empty vector and, as expected in this case, there was no activation by any of the anti-CD20 antibodies (supplemental Figure 1A).
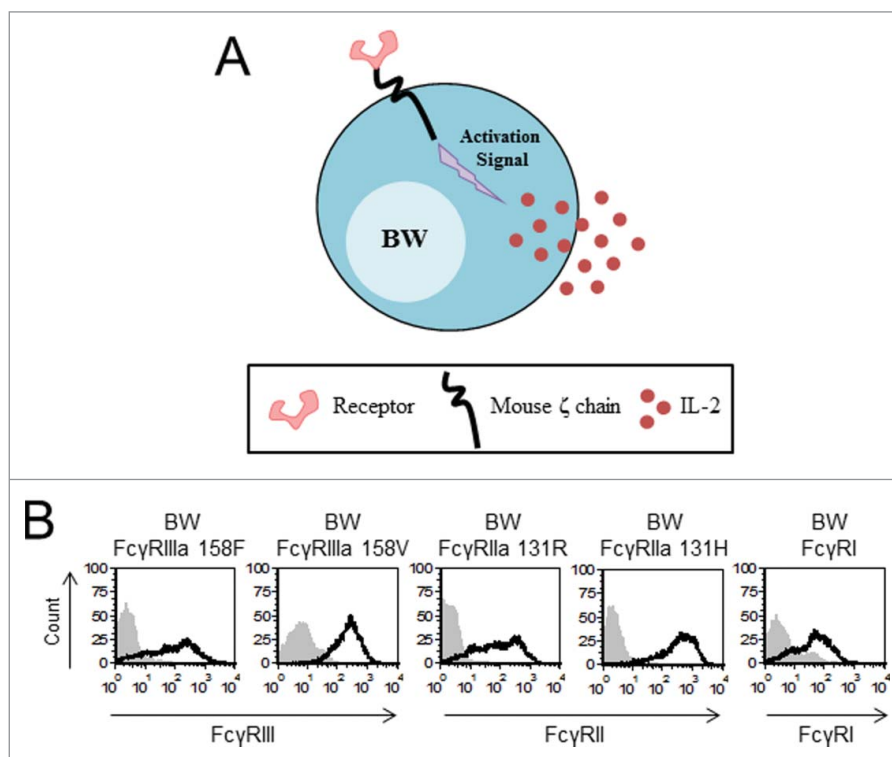
Figure 1. The FcγR-CD3ζ reporter system. (A) Schematic representation of the BW reporter system. BW5147 cells were stably transfected with the extracellular portion of different human Fcγ receptors fused to the transmembrane and cytoplasmic domains of mouse CD3ζ chain. Activation of a specific FcγR-CD3ζ results in secretion of mIL-2. (B) Each of the transfected BW cells was stained with a specific mAb against the particular FcγR it expresses (black histograms). Gray shaded histograms represent the background staining with an isotype-matched control antibody. One representative experiment is shown out of three performed.

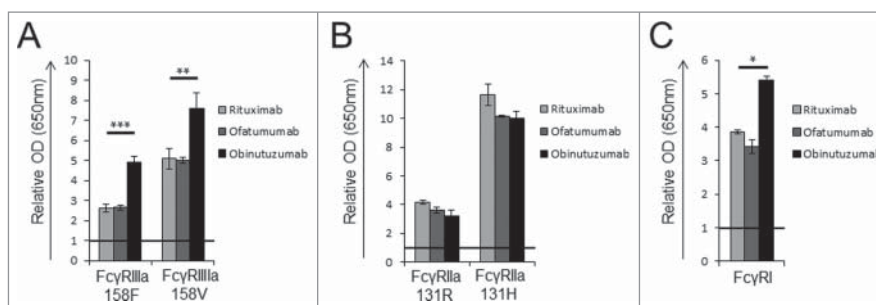**Figure 2.** Activation of FcγRs-CD3ζ by anti-CD20 antibodies bound to Raji cells. (A-C) Raji cells were pre-incubated with each of the three anti-CD20 antibodies (rituximab, ofatumumab and obinutuzumab). Subsequently, the target-bound antibodies were incubated with the various BW cells, which express the indicated FcγR-CD3ζ: FcγRIIIa (158F and 158V, A), FcγRIIa (131R and 131H, B) and FcγRI (C). The level of mIL-2 in the supernatant was determined by ELISA and normalized to the mIL-2 level of the control cells (BW transfectants incubated with untreated Raji cells). The mIL-2 level of the control cells was defined as one and is indicated by a horizontal black line. One representative experiment is shown out of two (in the case of FcγRIIIa) or four (in the case of FcγRIIa and FcγRI) performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test. Error bars represent standard deviation of triplicates.

Next, we tested two additional human Fcγ receptors. No major changes were noted in the activation of FcγRIIA (Fig. 2B, both low and high-affinity variants). However, we found that obinutuzumab leads to enhanced activation of FcγRI compared to rituximab and ofatumumab (Fig. 2C). To the best of our knowledge, the observation that obinutuzumab activates FcγRI more potently has not been previously reported.

As additional controls to these experiments we incubated the various BW reporter cells with the T cell line Jurkat, which does not express CD20, and, as expected, none of the anti-CD20 antibodies activated any of the FcγRs (supplemental Figure 1B).

Obinutuzumab leads to increased activation of FcγRIIIa and FcγRI in response to CLL cells

We next used CLL cells of human patients as target cells for our BW reporter cells. In addition to the wide use of rituximab in this disease, both ofatumumab and obinutuzumab are approved for treatment of CLL.[7,8]

Staining of CLL cells with the three anti-CD20 antibodies revealed reduced binding of obinutuzumab as compared to rituximab and ofatumumab as previously described (supplemental Figure 2).[12]

We pre-incubated each of the three anti-CD20 antibodies with CLL cells obtained from patients and tested the activation of the different FcγRs. The mIL-2 level of each patient sample was first normalized to the mIL-2 level of the control cells as above. Afterwards, the normalized results of cells from 15 CLL patients which were tested with a given FcγR-CD3ζ were averaged.

With CLL cells, as with Raji cells, we initially tested the activation of the two variants of FcγRIIIa by anti-CD20 antibodies. As expected, obinutuzumab demonstrated clear and significant advantages over the other two antibodies in activating both FcγRIIIa variants (Fig. 3A). The two variants of FcγRIIa were similarly activated by the three anti-CD20 antibodies upon interaction with the CLL samples (Fig. 3B).

Next, we tested the activation of FcγRI by the three anti-CD20 antibodies pre-incubated with CLL patient samples.

Importantly, obinutuzumab activated FcγRI more potently than the other two antibodies (Fig. 3C). The advantage of obinutuzumab in activating FcγRI was noted in each patient we analyzed separately (supplemental Figure 3A). Although most of our samples included cells from untreated CLL patients, the effect was demonstrated also in patients who were treated in the past or currently being treated for their disease (n = 4). Regardless of the relative advantage of obinutuzumab in activating FcγRI, the variability between the patients in activating this Fc receptor (supplemental Figure 3A) and possibly other Fc receptors by anti-CD20 antibodies should be explored in future studies.

We repeated these experiments under different conditions of incubation time, antibody concentration and effector to target (E:T) ratio. Regardless of the experimental conditions, obinutuzumab was superior in activating FcγRI (supplemental Figure 3B).

Obinutuzumab induces efficient antibody-dependent cellular cytotoxicity (ADCC) by primary NK cells

To test the functional relevance of the findings by the BW reporter system, we initially focused on FcγRIIIa. For that we used primary bulk NK cells since NK cells are activated mainly by this FcγR.

We isolated NK cells from two donors which express the low-affinity (158F) or the high-affinity (158V) variants of FcγRIIIa. These variants were identified by genotyping (see supplemental methods) as well as by staining with two different antibodies against FcγRIIIa. The low-affinity variant of FcγRIIIa was stained only with the 3G8 antibody clone (Fig. 4A), whereas the high-affinity variant of FcγRIIIa was stained with both 3G8 and MEM154 antibodies (Fig. 4B).

CLL cells obtained from patients were incubated with each of the three anti-CD20 antibodies or with a control antibody. Then, primary NK cells were added and the extent of NK cell degranulation was assessed by quantifying the percent of CD107a⁺ NK cells. In agreement with the BW reporter assay, incubation of the CLL cells with obinutuzumab induced more degranulation of NK cells as compared to rituximab and ofatumumab, and the results were similar with both variants of FcγRIIIa (Fig. 4C and 4D).
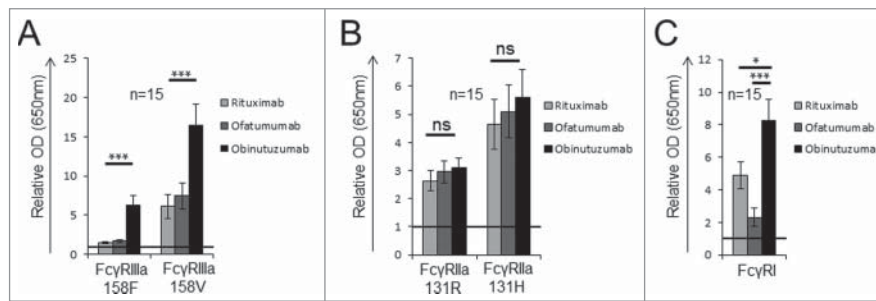
Figure 3. Activation of FcγRs-CD3ζ by anti-CD20 antibodies bound to CLL cells. (A-C) CLL cells were pre-incubated with each of the three anti-CD20 antibodies (rituximab, ofatumumab and obinutuzumab) and then with the transfected BW cells which express the indicated FcγR-CD3ζ: FcγRIIIa (158F and 158V, A), FcγRIIa (131R and 131H, B) and FcγRI (C). The level of mIL-2 in the supernatant was determined by ELISA. The activation level of a specific FcγR in each patient was normalized to the control cells (no antibody) and then averaged between all CLL patients (n = 15). The mIL-2 level of the control cells was defined as one and is indicated by a horizontal black line. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant; Student's t test. Error bars represent the standard error of the means of 15 patients (each in triplicates).

Stronger activation of FcγRI by obinutuzumab results in enhanced elimination of CLL cells by primary macrophages

To test the functional relevance of FcγRI activation by obinutuzumab, we performed phagocytosis assays with primary macrophages which are known to express this FcγR. Primary macrophages were differentiated from peripheral blood mononuclear cell (PBMCs) by selecting adherent cells and then culturing them with human serum. We examined the purity of the macrophages by analyzing the surface expression of CD14 (Fig. 5A). As expected, these primary macrophages expressed several FcγRs including FcγRI (Fig. 5B).

For the phagocytosis assay, CLL cells were first labeled with CFSE and then incubated with each of the three anti-CD20 antibodies. Primary macrophages were then added and co-incubated for 24 hours. Wright staining of the cells after this incubation period revealed several CLL cells inside macrophages (Fig. 5C).

In order to quantify the effect of each of the antibodies we used flow cytometry to assess the percentages of CFSE^{pos} cells in each of the experimental conditions (see methods and[15]). Incubation with obinutuzumab clearly enhanced the elimination of CLL cells as compared to incubation with rituximab (Fig. 5D).

To examine the role of FcγRI, we blocked FcγRI on the macrophages and repeated the phagocytosis assay. Blocking of FcγRI significantly reduced the elimination of CLL cells by obinutuzumab (Fig. 5E). In parallel, we tested a defucosylated variant of rituximab which has enhanced ability to activate FcγRIIIa but not FcγRI compared to WT-rituximab (see below). This enabled us to scrutinize the additive effect of the improved activation of FcγRI by obinutuzumab, and indeed obinutuzumab outperformed this rituximab variant (Fig. 5E). We therefore concluded that activation of FcγRI by obinutuzumab is functional.

Mechanism of action

To investigate why obinutuzumab can efficiently activate FcγRI, we initially studied the glycosylation properties of this antibody. Obinutuzumab has been glycoengineered so that its
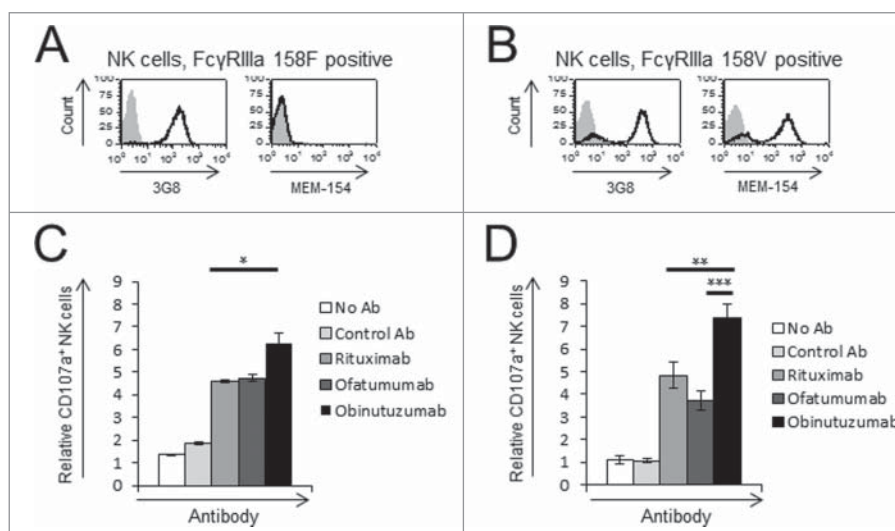

Figure 4. Obinutuzumab induces increased ADCC. (A-B) Primary NK cells were stained with two different antibodies against FcγRIIIA: 3G8 and MEM-154 (black histograms). The figure shows NK cells expressing the low-affinity (158F, A) and high-affinity variant (158V, B) FcγRIIIa. Gray filled histograms represent background staining with an isotype-matched control antibody. (C-D) CLL cells were pre-incubated with no antibody, with three different anti-CD20 antibodies or with a control antibody and then incubated with primary NK cells which express the low (C) or high (D) affinity variants of FcγRIIIa. Degranulation of NK cells was assessed by calculation of the ratio of CD107a+ NK cells out of the total NK cells (analyzed by flow cytometry). The ratio of CD107a+ NK cells in each experiment was normalized to the basal ratio of CD107a+ NK cells (NK cells only). The CD107a degranulation assays were performed with CLL cells of the same patient and were repeated with CLL cells of a different patient. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test. Error bars represent standard deviation of triplicates.
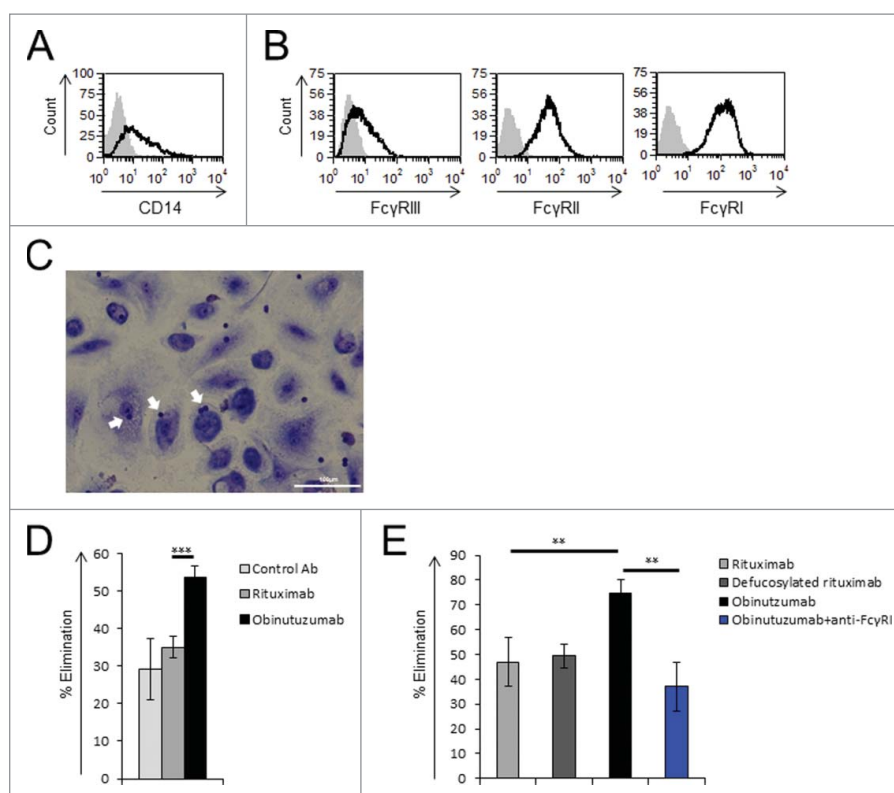
Figure 5. Obinutuzumab induces increased elimination of CLL cells by primary macrophages. (A) CD14 expression on primary macrophages, black histogram. Gray filled histogram represents background staining with an isotype-matched control antibody. (B) Expression of the indicated FcγRs on primary macrophages, black histograms. Gray filled histograms represent the background staining with an isotype-matched control antibody. (C) Wright staining of macrophages which were incubated with CLL cells and obinutuzumab (E:T 1:1). White arrow heads: CLL cells inside macrophages. Original magnification, x40, Scale bar 100 μM. (D-E) Quantification of the elimination of CLL cells by primary macrophages. CLL cells were stained with CFSE, pre-incubated with the indicated anti-CD20 antibodies and then co-incubated with primary macrophages. The concentration of CSFE^{pos} cells which remained in the supernatant was assessed by flow cytometry, normalized to control cells and its complement value to 100% was defined as the elimination ratio. (E) CLL cells were incubated with different anti-CD20 antibodies: rituximab, defucosylated rituximab and obinutuzumab, and then with primary macrophages. The phagocytosis assay with obinutuzumab was performed in parallel also with pre-blocking of FcγRI on the macrophages prior to their incubation with CLL cells. Error bars represent standard deviation of triplicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test. One representative experiment is shown out of two performed.

Fc segment has been defucosylated in order to increase the affinity of this antibody to FcγRIIIa.[12] Therefore we first tested whether our BW reporter system is able to corroborate this finding by comparing the activation of FcγRIIIa between rituximab and a non-fucosylated variant of rituximab (rit-hIgG1fut). As expected, the non-fucosylated form of rituximab activated more potently the two variants of FcγRIIIa compared to WT-rituximab (Fig. 6A). Next, we tested the importance of fucosylation of the Fc segment in activating FcγRI. As shown before, obinutuzumab activated FcγRI more potently compared to ofatumumab and rituximab (Fig. 6B). Importantly, however, and in contrast to the results obtained with FcγRIIIa (Fig. 6A), the defucosylated form of rituximab (rit-hIgG1fut) did not enhance the activation of FcγRI as compared to WT-rituximab (Fig. 6B).

We also used our reporter system to study the effect of the isotype on the activation of FcγRI by testing a panel of different variants of rituximab which includes human and mouse isotypes (supplemental Figure 4). The only isotype which activated FcγRI in a significant manner was mIgG2a (rit-mIgG2a). However, since the three anti-CD20 antibodies studied here have the same isotype, this cannot account for the observed differences. We also examined the possibility that a difference in the Fc sequences of rituximab and obinutuzumab might affect the FcγRI activation and found them to be identical (supplemental Figure 5).

Next, we tested the possibility that the increased activation of FcγRI is related to internalization of the anti-CD20 antibodies. It has been reported that obinutuzumab (a type II antibody), is comparatively less internalized upon binding to CD20.[13,14] We tested the internalization of anti-CD20 antibodies by comparing the CD20 staining on CLL cells using the various anti-CD20 antibodies, at 4°C (where internalization is not expected) and 37°C (where internalization can occur), along different time points (Fig. 6C). This experiment revealed significant internalization of rituximab and ofatumumab, but not obinutuzumab (Fig. 6C).

It was previously reported that the internalization of anti-CD20 antibodies is mediated through bridging of FcγRIIb and CD20[13]. To investigate the role played by FcγRIIb, we initially confirmed that FcγRIIb is expressed on CLL cells (Fig. 6D, boxed) and then we blocked it. Blocking of FcγRIIb on CLL cells significantly reduced the internalization of rituximab and ofatumumab (Fig. 6D).

Next, we examined the role played by FcγRIIb in FcγRI activation. To this aim, FcγRIIb was pre-blocked on CLL cells and then the CLL cells were incubated, as before, with the different anti-CD20 antibodies and subsequently with the BW-FcγRI cells (Fig. 6E). Pre-blocking of FcγRIIb on CLL cells diminished the internalization of rituximab and ofatumumab (Fig. 6D).
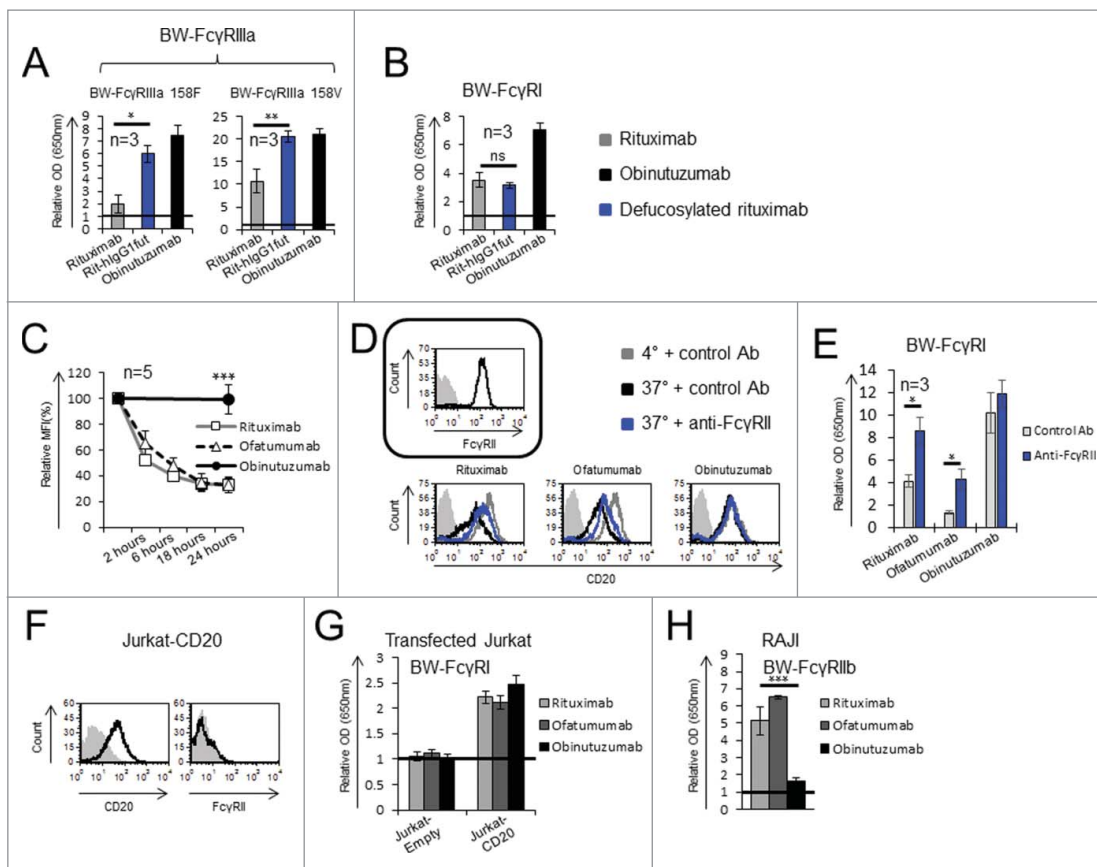
Figure 6. Activation of FcγRI by obinutuzumab is related to less FcγRIIb-mediated internalization. (A,B) CLL cells of three patients were pre-incubated with different anti-CD20 antibodies and then with transfected BW cells which express Fcγ-CD3ζ: FcγRIIIa (A) and FcγRI (B). Rit-hIgG1fut is a defucosylated variant of rituximab. (C) Average staining of five CLL cells with different anti-CD20 antibodies along a time course of 24 hours. At each time point we calculated the relative MFI of a specific antibody at 37° compared to the MFI of the same antibody at 4° after 2 hours. (D) Upper boxed: expression of FcγRII on CLL cells. Lower: staining of CLL cells with different anti-CD20 antibodies with pre-blocking of FcγRIIb on the CLL cells. The cells were incubated for one hour at 4° (gray), for 24 hours at 37° (black) or for 24 hours at 37° with pre-blocking of FcγRIIb (blue). (E) Activation of Fcγ-CD3ζ: FcγRI with different anti-CD20 antibodies which were incubated with CLL cells that were pre-blocked with control antibody (gray) or anti-FcγRII (blue). (F) Expression of CD20 and FcγRII on Jurkat cells which were transduced with CD20. CD20 was stained with rituximab. (G) Activation of Fcγ-CD3ζ: FcγRI with different anti-CD20 antibodies which were incubated with Jurkat cells transduced with an empty vector (left) or CD20 (right). (H) Activation of Fcγ-CD3ζ: FcγRIIb by different anti-CD20 antibodies which were incubated with Raji cells. For all experiments with the BW reporter cells the level of mIL-2 was determined by ELISA. The activation level of a specific FcγR in each patient was normalized to the control cells (defined as one and indicated as a black horizontal line) and then averaged between all CLL patients as indicated in each subplot. Error bars represent the standard error of the means of all patients tested (each in triplicates). For all flow cytometry experiments, gray shaded histograms represent the background staining with an isotype-matched control antibody. For all experiments which are not averaged results, one representative experiment is shown out of at least two performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's t test; ns – non-significant.

Pre-blocking of FcγRIIb on CLL cells significantly increased the activation of FcγRI by rituximab and ofatumumab, but not by obinutuzumab (Fig. 6E).

To further investigate whether the advantage of obinutuzumab in activating FcγRI is specific to cells expressing FcγRIIb, we over-expressed CD20 in Jurkat cells, which do not express FcγRIIb (Fig. 6F). We examined the activation of BW-FcγRI in response to the CD20-expressing Jurkat cells which were incubated with the different anti-CD20 antibodies and observed similar levels of activation by all three anti-CD20 antibodies (Fig. 6G).

Finally, to demonstrate that rituximab and ofatumumab, but not obinutuzumab can bind to FcγRIIb we used BW-FcγRIIb reporter cells, and found that obinutuzumab activates FcγRIIb significantly less than the other two antibodies (Fig. 6H).

Discussion

Here we employed a reporter assay to study the activation of human FcγRs by therapeutic anti-CD20 antibodies. Using this method we were able to corroborate previous reports regarding

the enhanced activation of FcγRIIIa by obinutuzumab. More importantly, we discovered that obinutuzumab activates FcγRI more potently than other anti-CD20 antibodies.

The assay we used is based on transfected BW cells, which express human FcγRs fused to CD3ζ. The main advantage of this assay is that it reports to what extent an FcγR is activated. Moreover, the activity of the antibodies is tested when they are bound to their natural ligand (in this case, CD20). Additional advantages of this system are that it is specific to a particular FcγR, sensitive and easy to use. The common methods which are currently available to investigate interactions between antibodies and FcγRs have several limitations. SPR measurements determine binding affinities rather than activation, and the two do not necessarily correlate.[16] In addition, functional tests with immune cells are complicated and primary immune cells usually express several Fc receptors which might confound the results.

Rituximab has dramatically improved the treatment of B-cell lymphoproliferative diseases and several autoimmune diseases. Even better results have been attained in CLL with the

novel anti-CD20 antibody obinutuzumab, yet this has yet to be confirmed in clinical trials using the same dose of both antibodies.[8,10] The reasons accounting for the apparent improved efficacy of obinutuzumab were not completely understood. Obinutuzumab is considered to be a type II anti-CD20 antibody as it does not accumulate CD20 in lipid rafts,[12] and this property has been associated with several other features, such as reduced recruitment of the complement system. In addition, obinutuzumab has been glycoengineered by defucosylation of asparagine 297 (Asn^{297}) in the Fc region,[12] in order to enhance the binding of this antibody to $Fc\gamma RIIIa$.[17,18] Indeed, affinity tests based on SPR demonstrated that obinutuzumab has increased affinity to both variants of $Fc\gamma RIIIa$. We verified these findings with our reporter assay both in a CD20 expressing cell line (Raji) and, more importantly, in patient-derived CLL cells. We also showed that this advantage of obinutuzumab results in enhanced ADCC of CLL cells by primary NK cells, in line with previous studies which were largely performed with cell lines as targets and sometimes with PBMC's as effectors.[12,19,20,21,22]

Whether obinutuzumab binds to $Fc\gamma Rs$ other than $Fc\gamma RIIIa$ has not been clearly elucidated. It was recently reported that obinutuzumab binds to $Fc\gamma RIIIb$ with higher affinity than does rituximab leading to enhanced activation of polymorphonuclear neutrophils (PMNs).[23] We have not assessed $Fc\gamma RIIIb$ using our system; however, its extracellular domain is highly homologous to that of $Fc\gamma RIIIa$.[24]

Here we show that obinutuzumab activates $Fc\gamma RI$ more potently than rituximab and ofatumumab using CLL cells as targets which leads to enhanced elimination of the CLL cells by macrophages. Contradictory results were previously reported regarding the level of antibody dependent cellular phagocytosis (ADCP) induced by these antibodies. This might be explained by different experimental conditions, especially incubation times and effector cells (mouse vs. human cells, which express different $Fc\gamma Rs$) as well as the different methods used to quantify the level of phagocytosis. Some studies, including in xenograft models, reported comparable levels of ADCP between obinutuzumab and rituximab.[19,25] However, other studies imply that obinutuzumab induces stronger ADCP as compared to rituximab, especially with longer incubation periods.[13,14,22]

We also studied the mechanism which explains the advantage of obinutuzumab in activating $Fc\gamma RI$. Antibody defucosylation *per se* is expected to affect the antibody binding solely to $Fc\gamma RIIIa$ and $Fc\gamma RIIIb$ since these $Fc\gamma Rs$ are the only ones which are glycosylated at a particular position of asparagine 162 (Asn^{162}).[26] Indeed, antibody defucosylation did not affect the binding to $Fc\gamma RI$[17] and glycoengineered obinutuzumab showed a comparable level of ADCP to that of the non-glycoengineered form of this antibody.[27] The results of our BW reporter system support these observations, since defucosylation of rituximab increased the activation of $Fc\gamma RIIIa$ but not of $Fc\gamma RI$.

We also tested the possibility that different levels of antibody internalization would explain the advantage of obinutuzumab. In recent years it has been reported that obinutuzumab undergoes less internalization as compared to rituximab and the internalization of rituximab is mediated through binding to $Fc\gamma RIIb$ on the surface of CLL cells.[13,14,22] We confirmed these

observations and, more importantly, found that blocking of $Fc\gamma RIIb$ increased the activation of $Fc\gamma RI$ by rituximab. In addition, we also showed that when CD20 is expressed in cells which do not express $Fc\gamma RIIb$, the advantage of obinutuzumab in activating $Fc\gamma RI$ is abolished. Finally, using our reporter system, we showed that obinutuzumab does not activate $Fc\gamma RIIb$, in contrast to rituximab.

Whether increased activation of $Fc\gamma RI$ is a unique property of obinutuzumab or a general feature of type II anti-CD20 antibodies should be better elucidated in the future. A critical obstacle in addressing this issue is that the isotype of other type II antibodies is murine. Despite this, and in agreement with our results, type II anti-CD20 antibodies have been reported to undergo less internalization compared to type I antibodies.[13,22] In addition, the type II antibody tositumomab has been shown to phosphorylate less $Fc\gamma RIIb$ as compared to rituximab,[13] and this was hypothesized to be a general property of type II antibodies.[6]

In summary, we established a novel reporter system to compare the activation of human $Fc\gamma Rs$ by anti-CD20 antibodies. Based on this method, we found that obinutuzumab leads to enhanced activation of $Fc\gamma RI$ compared to both rituximab and ofatumumab. We suggest that this assay will enable better evaluation of new therapeutic antibodies against CD20 or against other targets in general.

Materials and methods

Cells and antibodies

The cell lines used in this study were Raji, Jurkat (both human) and the mouse cell line BW5147.

The following anti-CD20 antibodies were used: rituximab and obinutuzumab (Roche), ofatumumab (GSK) and rituximab isotypes (anti-hCD20 isotype collection, InvivoGen). Details of the antibodies used are included in the supplemental methods.

Generation of transfected BW cells and the BW reporter assay

The extracellular domain of human $Fc\gamma RIIIa$ (CD16a), $Fc\gamma RIIa$ (CD32a), or $Fc\gamma RI$ (CD64) was fused to the transmembrane and intracellular domains of the mouse $CD3\zeta$ chain and cloned into the pcDNA3 vector. Details regarding the generation of the transfected BW cells are included in the supplemental methods.

For the BW reporter assay, we first incubated target cells with different anti-CD20 antibodies for one hour on ice. As target cells, we used mostly human CLL cells obtained from patients and also Raji or Jurkat cells. Raji and Jurkat cells were irradiated prior to their incubation with the antibodies (6000 rad). Then the various BW cells were added, and incubated at 37°C and 5% CO_2 for 48 hours in a final volume of 200 μl. In each well we incubated 50,000 BW cells and 50,000 target cells (in the case of Raji or Jurkat) or 200,000 target cells (in the case of CLL cells). In most of the experiments we used 0.5μg of the anti-CD20 antibodies per well (5μg/ml), except when using Raji cells and the two variants of $Fc\gamma RIIIa$, where we used 0.01 μg of the anti-CD20 antibodies per well (0.1 μg/ml). All cells were suspended in RPMI medium (Sigma-

Aldrich) supplemented with 1% glutamine, pyruvate, non-essential amino acids, penicillin-streptomycin (Biological industries) and 10% fetal bovine serum (FBS, Sigma-Aldrich), hereafter referred to as complete medium. The assay was performed in triplicates. After 48 hours, supernatants were collected, and the level of mouse interleukin-2 (mIL-2) was quantified by using anti-IL-2 mAbs and standard enzyme-linked immunosorbent assay (ELISA) (Powerwave XS Plate Reader, Biotek). As control antibodies for the anti-CD20 antibodies in the reporter assays we used trastuzumab (Roche) or human IgG1 isotype control. We performed the BW reporter assay under different experimental conditions including various incubation times, antibody doses and effector to target ratios.

NK cell purification and CD107a degranulation assay

Primary NK cells were isolated from the peripheral blood of healthy human volunteers, activated, and tested for purity as previously described.[28] Low and high-affinity FcγRIIIa-expressing donors were identified by genotype analysis as well as by FACS staining as described in the supplemental methods.

Details of the CD107a degranulation assay are included in the supplemental methods.

Macrophage extraction and phagocytosis assay

Macrophages were isolated and differentiated from the peripheral blood of healthy human volunteers by plastic adhesion (see for example[29]). Peripheral blood mononuclear cells (PBMCs) were cultured in 10-cm plates with serum free medium for two hours. Then the medium was replaced with complete medium supplemented with 10% human serum (H4522, Sigma-Aldrich). This medium was replaced every few days until receiving a confluent plate of adherent cells.

For the phagocytosis assay, macrophages were harvested with EDTA, and seeded in 8-well chamber slides (SPL) at a concentration of 50,000 cells/chamber. The phagocytosis assay was performed on the following day. CLL cells were stained with CFSE (CellTrace, catalog c34554, Thermofisher scientific) and then incubated with different anti-CD20 antibodies for one hour on ice. The stained CLL cells were added to the macrophages and incubated at 37°C and 5% CO_2 for 24 hours. We used 50,000 macrophages together with 50,000 target cells per chamber in a final volume of 200–250 μl. We used 0.5 μg (5 μg/ml) anti-CD20 antibody per chamber. For the blocking experiments, macrophages were pre-incubated for one hour with the anti-CD64 antibody (cat. 305002, BioLegend) at a dose of 1 μg (6.7 μg/ml). After 24 hours of incubation the medium of each chamber was extracted and the concentration of CFSE positive CLL cells in each chamber was assessed by flow cytometry. The reading of the cell concentration was performed in triplicates. For each chamber we calculated an elimination percentage which was defined as: $(1 - \frac{Concentration\ of\ CFSE^{pos}\ CLL\ cells\ (tested\ cells)}{Conentration\ of\ CFSE^{pos}\ CLL\ cells\ (control\ cells)}) \times 100$. As controls we used cells incubated with a control antibody or without any antibody. In addition, after extracting the medium the slides were rinsed, fixed and Wright stained (Hematek, Siemens).

Internalization assays and ectopic expression of CD20

For the internalization assays, CLL cells were incubated as mentioned above with anti-CD20 antibodies at 37°C and 4°C. For pre-blocking of FcγRIIb on the CLL cells we used anti-CD32 antibody (GTX74628, clone AT10) at a dose of 1 μg (10 μg/ml). A CD20 expressing plasmid was kindly provided by Professor Daniel Davis, the University of Manchester. CD20 was cloned into pHAGE-DsRED(−)-eGFP(+) lentiviral vector using a restriction free (RF) methodology.[30] Lentiviral virions were produced by transient three-plasmid transfection of 293T cells as previously described.[31] These viruses were used to transduce Jurkat cells and surface expression of CD20 was monitored by FACS.

Human samples

The collection of patient samples was approved by the institutional Helsinki committee of Hadassah Medical Center. Peripheral blood samples of CLL patients were collected in heparin tubes after obtaining informed consent. The samples were separated using a Ficoll gradient and the mononuclear fraction was aliquoted, cryopreserved in freezing solution containing 90% fetal bovine serum and 10% DMSO and later thawed for use.

Statistics

Excel and GraphPad Prism software version 7.02 were used for statistical analysis. For statistical tests we used Student's t test and ANOVA. A statistical test was considered significant when $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author contributions

References

1. Dotan E, Aggarwal C, Smith MR. Impact of Rituximab (Rituxan) on the treatment of B-Cell Non-Hodgkin's Lymphoma. P & T: A Peer-Reviewed J Formulary Management. 2010;35(3):148–157.

2. Gurcan HM, Keskin DB, Stern JN, Nitzberg MA, Shekhani H, Ahmed AR. A review of the current use of rituximab in autoimmune diseases. Int Immunopharmacol. 2009;9(1):10–25. doi:10.1016/j.intimp.2008.10.004. PMID:19000786.

3. National comprehensive cancer network. B-cell lymphomas (Version 7.2017). [accessed 2018 January 26]. https://www.nccn.org/professionals/physician_gls/pdf/b-cell.pdf.

4. Dierickx D, Kentos A, Delannoy A. The role of rituximab in adults with warm antibody autoimmune hemolytic anemia. Blood. 2015;125(21):3223–3229. doi:10.1182/blood-2015-01-588392. PMID:25827833.

5. Neunert C, Lim W, Crowther M, Cohen A, Solberg L, Jr, Crowther MA, American Society of Hematology. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood. 2011;117(16):4190–4207. doi:10.1182/blood-2010-08-302984. PMID:21325604.

6. Klein C, Lammens A, Schafer W, Georges G, Schwaiger M, Mossner E, Hopfner KP, Umaña P, Niederfellner G, et al. Epitope interactions of monoclonal antibodies targeting CD20 and their relationship to functional properties. MAbs. 2013;5(1):22–33. doi:10.4161/mabs.22771. PMID:23211638.

7. Cortelezzi A, Sciume M, Liberati AM, Vincenti D, Cuneo A, Reda G, Laurenti L, Zaja F, Marasca R, Chiarenza A, et al. Bendamustine in combination with ofatumumab in relapsed or refractory chronic lymphocytic leukemia: a GIMEMA Multicenter Phase II Trial. Leukemia. 2014;28(3):642–648. doi:10.1038/leu.2013.334. PMID:24220274.

8. Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, Chagorova T, de la Serna J, Dilhuydy MS, Illmer T, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. N Eng J Med. 2014;370(12):1101–1110. doi:10.1056/NEJMoa1313984.

9. Radford J, Davies A, Cartron G, Morschhauser F, Salles G, Marcus R, Wenger M, Lei G, Wassner-Fritsch E, Vitolo U, et al. Obinutuzumab (GA101) plus CHOP or FC in relapsed/refractory follicular lymphoma: results of the GAUDI study (BO21000). Blood. 2013;122(7):1137–1143. doi:10.1182/blood-2013-01-481341. PMID:23843495.

10. Cartron G, Watier H. Obinutuzumab: what is there to learn from clinical trials? Blood. 2017;130(5):581–589. doi:10.1182/blood-2017-03-771832. PMID:28584136.

11. Cragg MS, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. Blood. 2004;103(7):2738–2743. doi:10.1182/blood-2003-06-2031. PMID:14551143.

12. Mossner E, Brunker P, Moser S, Puntener U, Schmidt C, Herter S, Grau R, Gerdes C, Nopora A, van Puijenbroek E, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. Blood. 2010;115(22):4393–4402. doi:10.1182/blood-2009-06-225979. PMID:20194898.

13. Lim SH, Vaughan AT, Ashton-Key M, Williams EL, Dixon SV, Chan HT, Beers SA, French RR, Cox KL, Davies AJ, et al. Fc gamma receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy. Blood. 2011;118(9):2530–2540. doi:10.1182/blood-2011-01-330357. PMID:21768293.

14. Beers SA, French RR, Chan HT, Lim SH, Jarrett TC, Vidal RM, Wijayaweera SS, Dixon SV, Kim H, Cox KL, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for

15. Overdijk MB, Verploegen S, Bogels M, van Egmond M, Lammerts van Bueren JJ, Mutis T, Groen RW, Breij E, Martens AC, Bleeker WK, et al. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. MAbs. 2015;7(2):311–321. doi:10.1080/19420862.2015.1007813. PMID:25760767.

16. Corrales-Aguilar E, Trilling M, Reinhard H, Merce-Maldonado E, Widera M, Schaal H, Zimmermann A, Mandelboim O, Hengel H. A novel assay for detecting virus-specific antibodies triggering activation of Fcgamma receptors. J Immunol Methods. 2013;387(1–2):21–35. doi:10.1016/j.jim.2012.09.006. PMID:23023090.

17. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem. 2002;277(30): 26733–26740. doi:10.1074/jbc.M202069200. PMID:11986321.

18. Niwa R, Satoh M. The current status and prospects of antibody engineering for therapeutic use: focus on glycoengineering technology. J Pharmaceutical Sci. 2015;104(3):930–941. doi:10.1002/jps.24316.

19. Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T, Muth G, Ziegler-Landesberger D, Van Puijenbroek E, Lang S, et al. Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models. Mol Cancer Ther. 2013;12(10):2031–2042. doi:10.1158/1535-7163.MCT-12-1182. PMID:23873847.

20. Kern DJ, James BR, Blackwell S, Gassner C, Klein C, Weiner GJ. GA101 induces NK-cell activation and antibody-dependent cellular cytotoxicity more effectively than rituximab when complement is present. Leukemia Lymphoma. 2013;54(11):2500–2505. doi:10.3109/10428194.2013.781169. PMID:23452151.

21. Terszowski G, Klein C, Stern M. KIR/HLA interactions negatively affect rituximab- but not GA101 (obinutuzumab)-induced antibody-dependent cellular cytotoxicity. J Immunol. 2014;192(12):5618–5624. doi:10.4049/jimmunol.1400288. PMID:24795454.

22. Tipton TR, Roghanian A, Oldham RJ, Carter MJ, Cox KL, Mockridge CI, French RR, Dahal LN, Duriez PJ, Hargreaves PG, et al. Antigenic modulation limits the effector cell mechanisms employed by type I anti-CD20 monoclonal antibodies. Blood. 2015;125(12):1901–1909. doi:10.1182/blood-2014-07-588376. PMID:25631769.

23. Golay J, Da Roit F, Bologna L, Ferrara C, Leusen JH, Rambaldi A, Klein C, Introna M. Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. Blood. 2013;122(20):3482–3491. doi:10.1182/blood-2013-05-504043. PMID:24106207.

24. Nagarajan S, Chesla S, Cobern L, Anderson P, Zhu C, Selvaraj P. Ligand binding and phagocytosis by CD16 (Fc gamma receptor III) isoforms. Phagocytic signaling by associated zeta and gamma subunits in Chinese hamster ovary cells. J Biol Chem. 1995;270(43):25762–25770. doi:10.1074/jbc.270.43.25762. PMID:7592758.

25. Bologna L, Gotti E, Manganini M, Rambaldi A, Intermesoli T, Introna M, Golay J. Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B-chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. J Immunol. 2011;186(6):3762–3769. doi:10.4049/jimmunol.1000303. PMID:21296976.

26. Ferrara C, Stuart F, Sondermann P, Brunker P, Umana P. The carbohydrate at FcgammaRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. J Biol Chem. 2006;281(8):5032–5036. doi:10.1074/jbc.M510171200. PMID:16330541.

27. Herter S, Birk MC, Klein C, Gerdes C, Umana P, Bacac M. Glycoengineering of therapeutic antibodies enhances monocyte/macrophage-mediated phagocytosis and cytotoxicity. J Immunol. 2014;192(5):2252–2260. doi:10.4049/jimmunol.1301249. PMID: 24489098.

28. Yamin R, Lecker LS, Weisblum Y, Vitenshtein A, Le-Trilling VT, Wolf DG, Mandelboim O. HCMV vCXCL1 binds several chemokine

receptors and preferentially attracts neutrophils over NK cells by interacting with CXCR2. Cell Reports. 2016;15(7):1542–1553. doi:10.1016/j.celrep.2016.04.042. PMID:27160907.

29. Sabiiti W, Robertson E, Beale MA, Johnston SA, Brouwer AE, Loyse A, Jarvis JN, Gilbert AS, Fisher MC, Harrison TS, et al. Efficient phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis. J Clin Invest. 2014;124(5):2000–2008. doi:10.1172/JCI72950. PMID:24743149.

30. Unger T, Jacobovitch Y, Dantes A, Bernheim R, Peleg Y. Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. J Structural Biol. 2010;172(1):34–44. doi:10.1016/j.jsb.2010.06.016.

31. Elias S, Yamin R, Golomb L, Tsukerman P, Stanietsky-Kaynan N, Ben-Yehuda D, Mandelboim O. Immune evasion by oncogenic proteins of acute myeloid leukemia. Blood. 2014;123(10):1535–1543. doi:10.1182/blood-2013-09-526590. PMID:24449212.